

Full Length Research Paper

Comparative pharmacokinetic study of luteolin after oral administration of Chinese herb compound prescription JiMaiTong in spontaneous hypertensive rats (SHR) and Sprague Dawley (SD) rats

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JiMaiTong (JMT), a Chinese herb compound prescription consisted of *Flos chrysanthemi* Indici, *Spica prunellae* and *Semen cassiae* for anti-hypertension. Luteolin is one of the major bioactivity compositions in *F. chrysanthemi* Indici in JMT. There are some reports about pharmacokinetics of luteolin in extract of *F. chrysanthemi* and husks of peanut in normal rats, but it lacked pharmacokinetic information of luteolin residing in a Chinese herb compound prescription in hypertensive animal models. The present study aimed to develop a high-performance liquid chromatography with photodiode array detection (HPLC-DAD) method for determination of luteolin in rat plasma and for pharmacokinetic study after oral administration of JMT to spontaneous hypertensive rats (SHR) and normal Sprague Dawley (SD) rats. After oral administration of JMT to SHR and SD rats, respectively the content of luteolin in blood samples at different time points were determined by a reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with liquid-liquid phase extraction. This revealed a difference in disposition of luteolin in SHR and SD rats and a relationship between plasma concentration and hypertension of pathological state within the organism. This was the first report on the pharmacokinetic compare of luteolin between normal and hypertension pathological state rats. The hypertension pathological state would effects the disposition of luteolin, and it may provide a meaningful basis for the clinical application of JMT.

Key words: Luteolin, pharmacokinetic, *Flos chrysanthemi* Indici, hypertension, Chinese herb.

INTRODUCTION

JiMaiTong (JMT) is a Chinese herb compound prescription which can effectively depress the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) of spontaneous hypertensive rats (SHR) (Lv et al., 2010), map variability of renal

hypertensive rats promote the microcirculation blood flow of RHR by improving nitrogen oxide (NO) content and depress ET-1 and Ang II level in serum (Chen et al., 2012). JMT is composed of *Flos chrysanthemi* Indici, *Spica prunellae* and *Semen cassia* and contains flavonoids

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compositions such as luteolin, linarin etc. Luteolin (3', 4', 5, 7-tetrahydroxyflavone) is one of the major bioactivity compositions in JMT; it acts as anti-hypertension by relaxing the vascular smooth muscle (Kim et al., 2006; Jiang et al., 2005; Duarte et al., 1993; Ichimura et al., 2006) and blood vessel (Jiang et al., 2005), promotes the opening of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Hou et al., 2008) and protects the cardiac muscle (Lv et al., 2010). Since JMT is a candidate drug for anti-hypertension, it is essential to clarify the pharmacokinetics processes of luteolin *in vivo*.

The reaction of an organism to a similar traditional Chinese medicine would be different under the different physiological or pathological status (Zhao and Zhou, 2008) and the physiological disposition of the compositions in the drug would be changed as the same (Yang et al., 2005; Reng et al., 2006). These would induce the change of the plasma concentration and impact the pharmacological effects of the components. Therefore, a comparative study of pharmacokinetics of compositions in drug in pathological pattern animals and normal animals has more practical significance for determining the efficacy material and guide the clinical application.

There are some reports about pharmacokinetics of luteolin in normal rats after oral administration of *F. chrysanthem* extract or peanut shells extract and found that luteolin was present mainly as glucuronide conjugates in plasma and bile, and entacapone, a catechol-O-methyltransferase (COMT) inhibitor can increase the area under the curve (AUC) of luteolin when co-giving with *F. chrysanthem* extract (Wan et al. 2008, Chen et al. 2012, Chen et al. 2011). However there was lack of pharmacokinetic information of luteolin in Chinese herb compound in pathological pattern animals. The present study aimed to develop an improved quantitative method to determine luteolin in rat plasma using high-performance liquid chromatography with photodiode array detection (HPLC-DAD) and utilizing it in the pharmacokinetic study of the luteolin in SHR and Sprague Dawley (SD) rats after oral administration of JMT. The pharmacokinetic data could give us more information about the pharmacokinetics of JMT *in vivo* and give some reference for the clinical application.

MATERIALS AND METHODS

Chemicals

The reference standard of luteolin (>99% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Ministry of Health, Beijing, China). JMT was provided by the Institute of Material Medical, Zhejiang Chinese Medical University, China, containing 0.06% (w/w) luteolin determined by HPLC. Methanol was HPLC grade and the water was prepared by Milli-Q system (Millipore, Milford, MA, USA). Others were from standard commercial sources and were of the

highest quality available.

Animals

Male SHR rats weighing 280 to 320 g were obtained from Vital River Laboratory Animal Technology Co. Ltd and male SD rats weighing 260 to 280 g were obtained from Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were according to an approved animal use protocol of Zhejiang Chinese Medical University. They were housed in cages at $25 \pm 2^\circ\text{C}$ and exposed to a 12:12 h light-dark cycle with free access to food and water. Animals were fasted but had free access to water for 12 h before experiment and 2 h after drug administration.

Instrumentation

Analysis was performed using high-performance liquid chromatographic system Agilent 1200 series (Agilent Technologies, USA) equipped with an on-line vacuum degasser, a quaternary solvent delivery system, an auto-sampler, a diode array detector (DAD) and a homeothermic column compartment.

Chromatographic condition

The HPLC analysis was performed on an Ultimate XB - C18 column (250 mm \times 4.6 mm, 5 μm) with a mobile phase consisting of 5% glacial acetic acid and methanol (45:55, v/v) at $25 \pm 1^\circ\text{C}$, with a constant rate of 1 ml/min. The injection volume was 20 μl and the wavelength was set at 336 nm for quantitative analysis.

Stocking and working solution

Stock standard solution of luteolin (0.2 mg/ml) was prepared by dissolving 10.02 mg in methanol to 50 ml. A series of working solutions containing luteolin was prepared by subsequent dilution of the stock solution with methanol to 0.1, 1, 2, 5, 20, 100 $\mu\text{g/ml}$ and kept under 4°C .

Sample preparation

After oral administration of JMT, the blood samples were collected from the orbital venous sinus into heparinized tubes according to the specific schedule and then centrifuged at 4,000 g for 10 min at 4°C , the plasmas were collected and treated as the method of Ying et al. (2008), with some modifications. In brief, to detect the total form (free, glucuronidated, sulfated) of luteolin, a 500 μl plasma was hydrolyzed by 0.5 ml hydrochloric acid (10 M) at 80°C for 0.5 h in a 10 ml tube and then 8 ml mixed solution of acetic ether and N-hexane with proportion of 4:1 was added to the mixture. After ultrasonic for 5 min and vortex for 30 s, the tube was centrifuged for 10 min at 4,000 rpm. Then all the supernatant organic phase was carefully transferred to a 1.5 ml tube for eight times and evaporated to dryness with a nitrogen blowing concentrator at room temperature. The residue was reconstituted in 0.2 ml methanol and after centrifuged for 10 min at 12,000 rpm, the concentration of luteolin was determined by the HPLC method described.

Method validation

Specificity

Blank plasma from five rats, blank plasma spiked with luteolin and

sample obtained from rat after oral administration of JMT were processed and assayed as described in 2.6. Interference from endogenous or exogenous materials should not occur at the retention time of luteolin.

Linearity and range

Calibration standard was prepared as described in triplicate and the calibration curve ranging from 0.15 to 15.0 µg/ml was assessed by weighted (1/x) least squares linear regression based on plotting the peak area versus the concentration of the calibration standard. The lowest limit of quantitation (LLOQ) was defined as the lowest concentration that could be accurately and precisely quantitated corresponding to a signal-to-noise ratio of 3.

Precision and accuracy

Standard samples spiked with luteolin at low, medium and high concentrations (5, 20, and 100 µg/ml for all the analytes) were used for accuracy and precision studies (the end concentration was 0.75, 3, 15 µg/ml). Five replicates for each concentration were processed and analyzed as described for accuracy study. The assay recovery and extract recovery were calculated. The intra- and inter-day precisions (relative standard deviations, RSD) were evaluated by analyzing homogeneous samples in five replicates in 1 day. Intra- and inter-day precisions (RSD) were required to be less than 15%.

Stability

Stability of luteolin was evaluated by analyzing the standard sample at low, medium and high concentrations. Three replicates were stored at room temperature (about 25°C) for 12 h before sample processing. Sample concentrations were measured at the storage time of 2, 4, 6 and 12 h. The stability was assessed and expressed as remaining (%) of initial determined.

Pharmacokinetics study

3 male SHR and 3 male SD rats were fasted 12 h with free access to water, then a dose of 3.3 g/kg JMT (dissolved in water) was orally administered to SHR and SD rats, respectively. The blood samples were collected from the orbital venous sinus to heparinized tubes at pre-dose and post-dose at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 36 h and then centrifuged at 4,000 g for 10 min at 4°C. The plasma were collected and stored at -80°C until analysis.

Statistical analysis

Pharmacokinetic parameters were calculated with pharmacokinetic software phoenix WinNonlin 6.0. Measurements were expressed as the mean ± standard deviation and *t*-test was used to compare the measures of SHR and SD groups. *P* < 0.05 was considered statistically significant.

RESULTS

Specificity

The specificity of this method to plasma matrix was evaluated with plasma from five rats. The typical chromatograms

of (A) a blank plasma sample, (B) a blank plasma sample spiked with luteolin, (C) a plasma sample from a rat at 15 min after oral administration of 3.3 g/kg JMT are shown in Figure 1. No interferences from endogenous substances in rat plasma were observed at the retention times of luteolin (all samples were treated with hydrolysis preparation).

Linearity, range and sensitivity

The peak area of luteolin displayed a good linear relationship over the range of 0.15 to 15 µg/ml. The typical regression equations were as follows: $A = 48.12C + 0.57$. $R^2 = 0.9999$ (A: peak area of luteolin; C: concentration of luteolin in rat plasma). The LLOQ of all analytes was 0.075 µg/ml.

Precision and accuracy

Recovery, precision and accuracy data are presented in Table 1. The extract recoveries for luteolin were 79.4 to 95.8% and the assay recoveries of luteolin were 88.4 to 104.0%. The intra-day precision (RSDs) for luteolin was less than 3.7% and the inter-day precision (RSDs) was less than 6.5%.

Stability

The stability results of luteolin in rat plasma as shown in Table 1 which revealed the luteolin in stocking solution at 25°C for 12 h was stable (the remaining of the initial determined were > 97.0%).

Pharmacokinetic study

The method was successfully applied to analysis of plasma obtained from SHR and SD rats following a single oral dose of 3.3 g/kg JMT. Concentration-time profiles for luteolin of SHR and SD rats after giving JMT is shown in Figure 2. The plasma concentration of luteolin in SHR has double hump and was significantly higher than that of SD rats and the first class elimination rate in SHR was also higher (Table 2).

DISCUSSION

With the characters of moderate and multi-target effects, TCMs are used to treat chronic diseases such as hypertension, either in the form of single or combined herbs. The therapeutic activity of each herb is based on a complex combination and interaction of its various ingredients, showing an integral effect from those ingredients. The

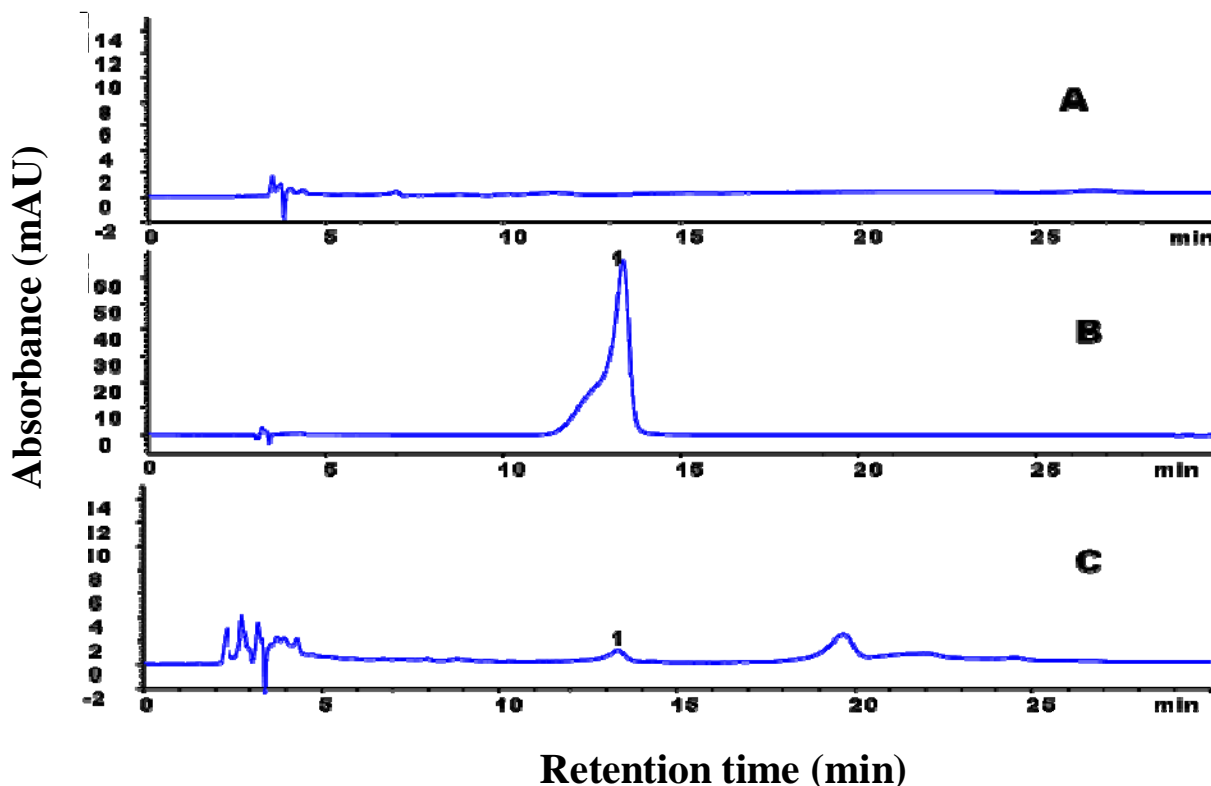


Figure 1. HPLC chromatograms of luteolin in rat plasma .

A: blank plasma; B: luteolin standard (show as 1, the retention time is 13.371min); C: plasma sample 15min after oral administration of 3.3g/kg JMT to SHR. All samples above were treated with hydrolysis.

Table 1. Precision, accuracy and stability of the method (mean \pm SD, n = 5).

Spiked conc. ($\mu\text{g/ml}$)	Intra-day		Inter-day		Assay recovery		Extract recovery		Stability (%)
	Measured conc. ($\mu\text{g/ml}$)	RSD (%)	Measured conc. ($\mu\text{g/ml}$)	RSD (%)	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)	
5	4.3 \pm 0.11	2.5	4.6 \pm 0.24	5.3	88.4 \pm 7.92	8.96	79.4 \pm 6.99	8.80	99.5 \pm 2.16
20	19.4 \pm 0.71	3.4	20.4 \pm 1.33	6.5	102.6 \pm 8.78	8.56	82.8 \pm 7.06	8.53	98.1 \pm 0.77
100	103.9 \pm 3.53	3.7	103.0 \pm 6.60	6.4	104.0 \pm 4.08	3.93	95.8 \pm 3.76	3.92	97.0 \pm 3.00

organism is a carrier of medicines to produce a marked effect and an object for drug treatment (Zhang et al., 2008). The pharmacodynamic actions or toxicity of ingredients is closely related with organism state (Ni and Zhang, 2009). Different states of the organism have different response to a medicine and it is important to carry researches on the pharmacokinetic of ingredients in organism with healthy or pathological state (Tian et al., 2012). The luteolin is one of the main active ingredients in the JMT and is important and responsible for the integral effect of JMT on anti-hypertension. The pharmacokinetic study of luteolin in rats with healthy or hypertension state would make for an objective pharmacodynamic evaluation

of JMT.

The adopted RP-HPLC method has been developed and successfully applied to the pharmacokinetic analysis of bioactive components from TCM. Thus, with some modifications, the well-developed RP-HPLC coupled with liquid-liquid phase extraction can be considered as a suitable method for this study. However, there are complicated compositions in the Chinese herb compound prescription JMT, so an optimized chromatographic condition was very important in the present study. In this study, an Ultimate XB-C₁₈ column (250 mm \times 4.6 mm, 5 μm) was applied to separate the luteolin. The mixtures of 5% glacial acetic acid and methanol or 0.5% phosphoric

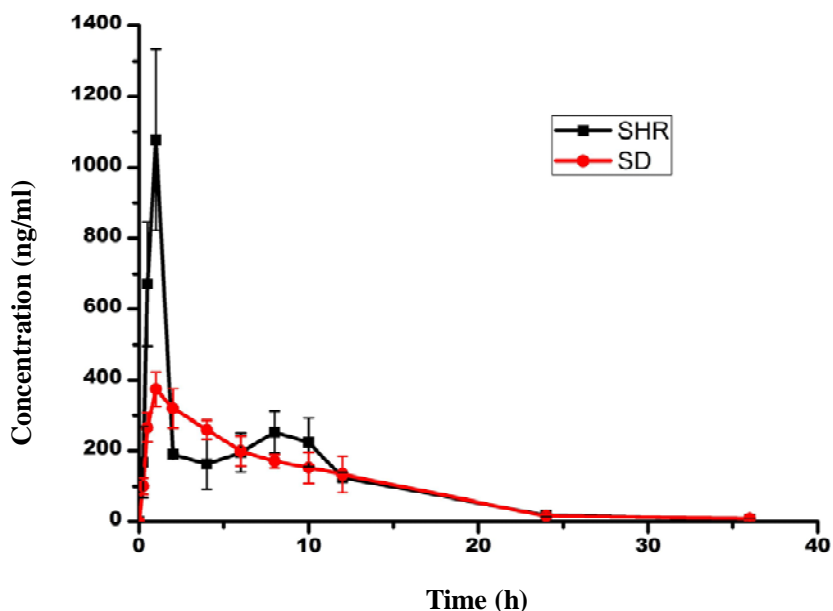


Figure 2. Mean-plasma concentration-time profiles of luteolin after oral administration of 3.3 g/kg JMT to SHR and SD rats. Data were expressed as mean \pm SD, $n=3$.

Table 2. Main pharmacokinetic parameters of luteolin in SHR and SD rats after oral administration of 3.3g/kg JMT (mean \pm SD, $n = 3$).

Parameter	Animals	
	SHR	SD
Lambda_z (1/h)	0.173 \pm 0.03*	0.119 \pm 0.03
HL_Lambda_z t _{1/2} (hr)	4.08 \pm 0.58	6.14 \pm 1.59
T _{max} (h)	1.00 \pm 0.00	1.33 \pm 0.58
C _{max} (μ g/ml)	1.08 \pm 0.26*	0.39 \pm 0.03
AUC _{last} (h μ g/ml)	4.19 \pm 0.55	3.63 \pm 0.67
AUC _{INF_obs} (h μ g/ml)	4.23 \pm 0.56	3.71 \pm 0.65
V _z _F_obs (L/kg)	4693.0 \pm 1,181	8254.4 \pm 3,595
Cl_F_obs (L/h/kg)	789.0 \pm 98	909.7 \pm 176
MRT _{last} (h)	7.38 \pm 0.70	8.03 \pm 0.51
MRT _{INF_obs} (h)	7.69 \pm 0.80	8.93 \pm 0.88

*P < 0.05 vs SD rats. Spontaneous hypertensive rats (SHR) and Sprague Dawley (SD) rats.

acid and methanol were compared to get a suitable mobile phase condition and the former was found to make a good separation.

After optimizing the proportion of methanol, a mobile phase of 5% glacial acetic acid and methanol (45:55, v/v) was determined. Luteolin has a free hydroxyl which could be further conjugated by UGTs and existed as a glucuronide form (Shimoi et al., 2001; Liu et al., 1995) and it is difficult to detect free luteolin in plasma; therefore a hydrolysis treatment with hydrochloric acid or α -glucuronidase of sample was very important to assay the methylated metabolites of luteolin *in vivo*. In this study,

hydrolysis treatment with hydrochloric acid was selected and after an optimization of extraction conditions with organic solvent for free luteolin, an organic solvent mixture of acetic ether and N-hexane (4:1, v/v) was determined.

In this study, non-compartment model was adopted to perform the evaluation of pharmacokinetics of luteolin in SD rats and SHR after oral administration. An obvious double peak was observed in SHR which indicated that in SHR, enterohepatic circulation may be the way of luteolin's re-absorption (Ying et al., 2008; Chen et al., 2007). As we know, the AUC reflect the relative amount of drug absorbed into the systemic circulation, the large

AUC means the more drug absorption and with the same dosage *in vivo*, the higher blood concentration means the smaller V_d value, which showed the drug is mainly distributed in blood and conversely in tissues. In this study, we found that the C_{max} ($1.08 \pm 0.26 \mu\text{g/ml}$), AUC ($4.19 \pm 0.55 \text{ h } \mu\text{g/ml}$) and λ_{z} (K_e , $0.173 \pm 0.03 \text{ 1/h}$) of luteolin in SHR were large than that in SD rats ($P < 0.05$), but T_{max} (1.0 h), V_d ($4693.0 \pm 1.181 \text{ L/kg}$) and MRT_{last} ($7.38 \pm 0.70 \text{ h}$) were smaller. Indicating that in SHR the luteolin after oral administration can be rapidly and effectively absorbed from SHR gastrointestinal tract into the blood and then rapidly eliminated and cleared from the body; while in SD rats, it was absorbed into the blood and widely transported from the blood into tissues and this course prolong the elimination.

Luteolin is a good substrate of catechol-O-methyltransferase (COMT) and *in vivo* it would be methylated to chrysoeriol and diosmetin by COMT (Chen et al., 2011). This metabolic character is contributed to the faster elimination of luteolin in rats (Chen et al., 2012). With the changing of activity of liver metabolic enzyme or the quantity of β -glucuronidase, the metabolism of compositions in the animal with pathological status would be changed (Deng et al., 2008; Liu et al., 2012). The pharmacokinetics difference of luteolin in SHR and SD rats observed from this study show that the hypertension pathologic state would affect the metabolism character of luteolin, but the mechanism for this was unclear and needs further investigation.

Taken as a whole, all available pharmacokinetic data indicated that luteolin displayed ideal pharmacokinetic profiles in SHR plasma after oral administration of JMT. Since the JMT is a candidate for the treatment of hypertension, it would be very useful and meaningful to obtain the main active ingredients, such as luteolin, pharmacokinetic information for the better understanding of the pharmacology. To some extent, our pharmacokinetic data presented a substantial evidence for the *in vivo* efficacy of luteolin in the organism with hypertension pathologic state, since a favorable absorption, distribution and elimination of this compound *in vivo* have been demonstrated.

In summary, together with our pharmacological findings, this study could provide some useful clues and guidance, such as dosage regimen and application strategy, for clinical application. The next step will be to clarify this compounds' tissue distribution both in SD rats and SHR and the underlying mechanism of action.

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